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(54) Title: METHOD FOR FREEZING ENGRAFTING CELLS

(57) Abstract

The present invention provides a method for preparing engrafting cells for future use, comprising the steps of (a) purifying engrafting cells; (b) concentrating the purified engrafting cells; (c) resuspending the concentrated cells to a concentration of about 10×10^6 to 40×10^6 cells/ml in a solution that substantially maintains cell viability during freezing; and (d) freezing the resuspended cells under a first set of conditions which substantially maintains cell viability.

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Description

METHOD FOR FREEZING ENGRAFTING CELLS

Cross-Reference to Related Application

This application is a continuation-in-part of pending U.S. Application Serial No. 07/513,543, which was filed April 23, 1990.

Technical Field

The present invention relates generally to methods for freezing cells and, more specifically, to methods for freezing engrafting cells.

Background of the Invention

Bone marrow transplantation (BMT) has emerged as the therapy of choice for patients with certain oncological or hematological diseases (Kamani *et al.*, "Bone marrow transplantation. Problems and prospects." *Med. Clin. North Amer.* 68:657-674, 1984). For cancer therapy, the dosage of conventional cytotoxic drugs and radiotherapy is limited because of toxicity to bone marrow stem cells. To overcome this difficulty, patients have been treated with high dose chemo- or radiotherapy followed by allogenic bone marrow transplantation (BMT) as a rescue. Although allogenic BMT has markedly improved the survival of patients with aplastic anemia, acute leukemia, and severe immunodeficiency, many difficulties have limited its clinical application. First, most patients do not have a histocompatible sibling donor and the feasibility of such transplantations has generally depended on the availability of a suitable donor. Second, the recipients should be treated with a pretransplant immunosuppression. Third, allogenic BMT can be associated with severe complications and morbidity caused by graft vs. host disease and infection.

Compared to allogenic BMT, autologous bone marrow transplantation (ABMT) has significant advantages. It has been used in combination with intensive chemotherapy to treat patients with various solid tumors where there is no involvement with bone marrow, including for example malignant lymphomas, melanomas, and carcinomas of the lung and breast. ABMT itself does not act directly on tumor cells, but can facilitate intensive chemotherapy and radiotherapy by reconstitution of immunological and

hematopoietic activities (*see* Gorin *et al.* "Chemotherapy and autologous bone marrow transplantation in acute leukemias, malignant lymphomas and solid tumors," *Eur. J. Cancer 17*:557-568, 1981). Recently, ABMT has been commonly used to treat patients with leukemia, including acute lymphocytic and nonlymphocytic leukemia, and chronic granulocytic leukemia in the blastic phase, as well as patients with lymphoma and breast cancer.

Due to the benefits associated with chemotherapeutic or irradiation therapies, followed by bone marrow reconstitution, preservation of marrow has received considerable scientific attention. However, in order to obtain the benefits of ABMT, the marrow must be stored for a period of time to allow chemotherapy or radiation treatment of the patient. Early work on storing marrow showed that storage at room temperature or 4°C led to a rapid loss of the engrafting cells. Thus a delay in reinfusion of the marrow of more than several days could lead to failure of the marrow to re-engraft in the patient. Complications sufficient to delay reinfusion are common among advanced cancer patients, which have made this storage method untenable. Several scientists have since shown that whole bone marrow or semi-purified preparations such as buffy coats can be frozen, thawed, and reinfused into patients successfully after long storage times.

One major difficulty with current methods, however, is that only relatively low percentages of the actual cells which engraft or reconstitute the bone marrow survive the cryopreservation process. For example, Lemoli *et al.* (*Haematologica 73*:101-104, 1988), collected whole bone marrow and separated out a mononuclear fraction. These cells were frozen, but only 50% to 66% of CFU-GM cells were recovered upon thawing. Figuera *et al.* (*Cryobiology 23*:470-475, 1986) utilized a controlled rate freezer to freeze marrow buffy coats in storage bags, but only recovered about 36% to 43% of the CFU-GM cells. Visani *et al.*, *Crybiology 20*:587-590 (1983), froze marrow buffy coats in a controlled rate freezer, and only recovered about 70% of cells following cryopreservation.

In addition, present cryopreservation strategies generally utilize large quantities of DMSO in order to protect the cells during freezing and thawing. For example, patients receiving semi-purified bone marrow typically also receive 10 to 20 ml of DMSO, while patients who are given whole bone marrow may receive as much as 150 ml of DMSO.

Administration of large qualities of DMSO may have a toxic effect on patients who are already weakened by chemotherapy or irradiation. Patients who have been given a large quantity of DMSO, in addition to experiencing

unpleasant breath and odor, frequently also experience headaches, nausea, chills, dizziness and vomiting. Additionally, there is a risk of more serious problems including heart arrhythmias, lesions of the eye, hypertension, renal failure, and even death.

Finally, current methods of cryopreserving bone marrow often lead to cell aggregates and lysis of red blood cells. These aggregates and the hemoglobin released by the lysed red blood cells are implicated in pulmonary distress and renal failure, which is occasionally noted in bone marrow recipients.

The present invention overcomes the toxic effects of DMSO cell aggregates and lysed red blood cells, and the poor cell recoveries of prior cryopreservation methods, and further provides other related advantages.

Summary of the Invention

Briefly stated, it is an object of the present invention to provide methods for preparing engrafting cells for future use. More specifically, engrafting cells may be prepared and frozen for extended periods of time, and later retrieved for therapeutic or research purposes.

Within one aspect of the invention, a method is provided for preparing engrafting cells for future use, comprising the steps of (a) purifying engrafting cells from a suitable blood product; (b) concentrating the purified engrafting cells; (c) resuspending the concentrated cells to a concentration of about 10×10^6 to 40×10^6 cells/ml in a solution that substantially maintains cell viability during freezing and thawing; and (d) freezing the resuspended cells under a first set of conditions which substantially maintain cell viability. Within a related aspect of present invention, a method for preparing engrafting cells for future use is provided, comprising the steps of (a) purifying engrafting cells from a suitable blood product; (b) concentrating the purified engrafting cells; (c) resuspending the concentrated cells in a solution that substantially maintains cell viability during freezing and thawing, the solution containing a total of about 0.002 ml to about 1 ml of DMSO; and (d) freezing the resuspended cells under a first set of conditions which substantially maintains cell viability. Within one embodiment of the present invention, the purified engrafting cells are resuspended to a concentration of about 10×10^6 to about 40×10^6 cells per ml.

Within one embodiment, the methods noted above further comprise, subsequent to the step of freezing, thawing the frozen cells under a second set of conditions which substantially maintain cell viability. Within a

preferred embodiment, the cells are thawed in a 37°C water bath, and then in a subsequent step, slowly diluted with a physiological buffer.

Within other embodiments of the present invention, the step of purifying comprises passing the blood product over an immunoaffinity column which purifies the engrafting cells, and the step of concentrating comprises centrifuging the engrafting cells.

In other embodiments, the concentrated cells are resuspended in a solution which contains about 6% HES, or a solution that contains a total of about 0.002 ml to about 1 ml of DMSO, or preferably, in a solution comprising media, protein, and a penetrating cryoprotectant. Particularly preferred media include RPMI 1640, TC 199, and Iscoves DMEM.

Within yet another embodiment, the step of freezing is accomplished by freezing the cells at a controlled rate. Within a presently preferred embodiment, the step of freezing at a controlled rate is accomplished by (a) cooling the cells down to about 4°C; (b) cooling down the 4°C cells at a rate of about one degree per minute until the cells reach about -4°C; (c) cooling down the -4°C cells at a rate of about 0.5 degrees per minute until the cells reach about -20°C; (d) cooling down the -20°C cells at a rate of about 1.0 degree per minute until the cells reach about -40°C; and (e) cooling down the -40°C cells at a rate of about 10.0 degrees per minute until the cells reach about -90°C.

Within another aspect of the present invention, a composition is provided, comprising (a) a therapeutic dose of engrafting cells produced according to the present invention, and (b) an aqueous solution containing a total of about 0.002 ml to about 1 ml of DMSO. Within one embodiment, a method is provided for treating immunocompromised patients comprising the step of administering to a patient such a composition. Administration may be performed through use of either a syringe or a drip bag which contains this composition.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a graph which illustrates the freezing of engrafting cells at a controlled rate.

Figure 2 is a bar graph which compares the viability, cell recovery, and colony-forming cell (CFC) recovery of cryopreserved purified engrafting cells with cryopreserved whole bone marrow.

Figure 3 is a bar graph which compares the viability, cell recovery, and CFC recovery of cryopreserved purified engrafting cells which are frozen at different cell concentrations.

Figure 4 is a bar graph which compares the viability, cell recovery, and CFC recovery of cryopreserved purified engrafting cells in 7.5% DMSO, and in 10% DMSO.

Detailed Description of the Invention

As noted above, the present invention provides methods for preparing engrafting cells for future use. Within the context of the present invention, the term "engrafting cells" includes totipotent hematopoietic stem cells as well as early progenitor cells such as colony-forming cells (CFCs). Representative examples of CFCs include CFU-E, CFU-G, CFU-M, CFU-GM, CFU-GEMM, and BFU-E cells. Given the fact that generally as many as one-third to two-thirds of CD 34 positive cells may be stem cells or colony-forming cells, it should be understood that when CD 34 cells are concentrated or purified, engrafting cells are similarly concentrated or purified.

Utilizing devices and methods which are described in more detail below, engrafting cells may be purified from various blood products, including for example, peripheral blood and whole bone marrow. For purposes of the present invention, engrafting cells are considered to be "purified" if at least 20% of the purified cells are CD 34 positive cells. Preferably, the CD 34 positive cells should be purified to greater than 90% purity. In addition, it is desirable to keep the total numbers of platelets, granulocytes, and red cells as low as possible in order to prevent clumping and the release of degradative enzymes which decrease engrafting cell recovery and viability. More specifically, it is generally desirable that the purified engrafting cells contain less than about 1% platelets, less than 50% and preferably less than about 25% granulocytes, and less than 10% and preferably less than about 1% red cells.

Purification of engrafting cells may be accomplished through use of a ligand which specifically recognizes antigens on these cells. For example, antibodies which specifically recognize the CD 34 antigens may be utilized in the devices and methods described below in order to purify engrafting cells. Representative examples of antibodies which specifically recognize the CD 34 antigen include MY-10 and HPCA2, (Becton-Dickinson, Mountain View, Calif.), QBEND-10 (Quantum Biosystems, Cambridge, U.K.) and 12.8 (CellPro®, Bothell, Wash.).

Various methods and devices may be utilized to purify engrafting cells, including the use of magnetic beads, panning, and flow cytometry (Fluorescence Activated Cell Sorting "FACS") (*see, for example*, U.S. Patent Nos. 4,714,680 and 4,965,204, hereby incorporated by reference). Particularly preferred methods and devices are immunoaffinity columns such as those which are described in U.S. Application Serial No. 07/513,543, entitled "Immunoselection Device and Method" (hereby incorporated by reference in its entirety). Briefly, this application describes methods and devices for isolating or separating target particles such as engrafting cells, from a mixture of non-target and target particles. Included within this application is a discussion of devices and methods wherein target particles are separated in a direct method by passing the particles through a column containing a bed of low nonspecific binding porous material which has a ligand capable of specifically binding to the target particles. Within one aspect of the application, a device is provided which generally comprises (a) a column having a proximal end with an inlet port through which fluid may enter the column and a distal end with an outlet port through which fluid may exit the column, (b) a bed of low nonspecific binding porous material within the column, the porous material having a biotin adsorbing group immobilized on the surface thereof, wherein the pores of the porous material are of a size sufficient to allow the biotin adsorbing group to enter into the pores, but not so large as to allow collapse of the bed, and wherein the interstitial spaces of the bed are of a size sufficient to allow the particles to flow through the bed. The device may further comprise a means, located within the column, for agitating the porous material upon the application of an external force, such that bound target particles are released from the porous material. Within other aspects of this application the target particles are separated by either a one-step or two-step method utilizing avidin and biotin. It should also be noted, however, that for purposes of the present invention other materials may be utilized within the immunoaffinity column, including for example non-porous materials.

A particularly preferred immunoaffinity column is described in pending U.S. Application (Attorney's Docket No. 200072.407) entitled "Improved Apparatus and Method for Cell Separation" (hereby incorporated by reference in its entirety). Briefly, within one aspect of this application, a "cell separator" is provided including a column assembly for separating target cells from a sample fluid, the column assembly including a column, a sample fluid supply bag and a fluid collection bag wherein the column is provided for receiving the sample fluid from the sample fluid supply bag and for separating the target cells from the

sample fluid and retaining the target cells, and wherein the fluid collection bag is provided for receiving the target cells after being released from the column, the cell separator comprising an agitation means for agitating the contents of the column to assist in releasing the sample cells retained in the column, the agitation means being responsive to a drive signal for varying amounts of agitation of the contents of the column to vary the rate at which the sample cells are released, column sensor means for providing a column signal indicative of the optical density of fluid flowing out of the column and into the fluid collection bag, a column valve means responsive to a column valve control signal for selectively enabling the fluid coming out of the column to flow into the fluid collection bag, and a data processor means for controlling the operation of the cell separator, the data processor means being responsive to the column signal for providing the drive signal and the column valve control signal to prevent inadequate concentrations of the target cells from being collected. One embodiment of this invention is the CEPRATE LC™ cell separation system which is available from CellPro® (Bothell, Wash.).

The purified engrafting cells are then concentrated. Various methods may be utilized to concentrate the purified cells, including for example sedimentation and filtration, although centrifugation is generally preferred. Within a preferred embodiment of the invention, the purified engrafting cells are concentrated by centrifugation at 150 x g for 10 minutes, and the supernatant is discarded.

The concentrated engrafting cells are then resuspended in a solution that substantially maintains cell viability during freezing and thawing. Within the context of the present invention, cell viability is "substantially maintained" if greater than 80%, and preferably greater than about 90% of the cells remain viable after freezing and thawing. A particularly preferred method for determining engrafting cell viability is described in greater detail below in Example 8.

Various solutions may be utilized in order to substantially maintain cell viability during freezing and thawing. These solutions maintain cell viability during freezing and thawing by (1) preventing or reducing the formation of intracellular ice crystals, (2) increasing osmotic pressure inside cells so that the volume reduction upon freezing is reduced, and (3) stabilizing the cell membrane. The basis of the solution may be composed of any physiologically acceptable fluid, including for example simple saline or buffered saline (*e.g.*, phosphate buffered saline or "PBS"), or cell culture media. Representative examples of cell culture

media suitable for use in the present invention include RPMI 1640, TC 199, or Iscoves DMEM (all available from Gibco BRL, Gaithersburg, Md.).

The solution should additionally contain a penetrating cryoprotectant, a membrane stabilizing agent, a protein, or preferably, all three. Penetrating cryoprotectants such as glycerol, DMSO, and formamide aid in the maintenance of cell viability by increasing the osmotic pressure inside the cells so that the volume reduction upon freezing is reduced. A particularly preferred penetrating cryoprotectant is pharmaceutical grade DMSO which is utilized at a final concentration of 4% to 20%, and preferably at a final concentration of 7.5% to 10%. Use of DMSO at these concentrations (given the cell concentrations discussed below) should result in the cells being resuspended in a solution containing a total of from about 0.002 ml to about 1 ml of DMSO. As discussed in more detail below, this low total dose of DMSO significantly improves the efficacy of therapeutically administering engrafting cells.

Membrane stabilizing agents are believed to aid in the maintenance of cell viability by helping to reduce cell damage due to dehydration during freezing. Examples of membrane stabilizing agents include hydroxyethyl starch (HES), polyvinyl pyrrolidone (PVP), and glucose. A particularly preferred membrane stabilizing agent is low molecular weight HES (American Critical Care, McGaw Park, Ill.), which may be utilized in the solution at a final concentration ranging from 4% to 9%, and preferably about 6%.

Proteins, as noted above, are believed to function by reducing cell damage due to dehydration during freezing in a manner similar to membrane stabilizing agents. Examples of suitable proteins include albumin (animal or human), hemoglobin, animal serum (*e.g.*, HYCLONE®, Logan, Utah), and human plasma (available from the Puget Sound Blood Center, Seattle, Wash., or from other blood centers, blood banks, or plasma centers). The protein may be utilized at a wide range of concentrations, ranging from about 5% to 90% in the solution. It should be noted, however, that if a protein solution is utilized along with a media containing Ca^{++} or Mg^{++} ions, it is preferable to include between 5 and 20 units of heparin per ml, and preferably about 10 units of heparin per ml of the solution in order to prevent clotting or coagulation of the cells by protein. A particularly preferred protein which is utilized in the present invention is human autologous plasma, because it avoids the possibility of contaminating engrafting cells with foreign viruses (for example, HIV), and overcomes compatibility difficulties which might arise if noncompatible serum was utilized (for example, fetal bovine serum). Within a preferred embodiment, autologous plasma is

generated by first centrifuging plasma obtained from a donor at 150 x g in order to remove cellular debris. Next, the supernatant is removed and centrifuged at 10,000 to 15,000 x g in order to remove particulate matter. The supernatant is then passed through a 0.8 μ filter, and then through a 0.2 μ filter in order to further clarify and sterilize the plasma. Within a preferred embodiment of the invention, autologous plasma is utilized at a final concentration of about 10% to 20% of the solution.

Additionally, defined nutrient supplements which have no protein or only minimal quantities of protein may also be utilized to reduce cell damage due to dehydration during freezing in place of a protein. A particularly preferred defined nutrient supplement is Ex Vivo.

As noted above, a preferred solution for use within the present invention comprises media, protein, and a penetrating cryoprotectant. In one embodiment, this solution comprises TC 199 media containing about 20% autologous plasma, and 7.5% DMSO.

It is also necessary to resuspend the cells in a volume of solution such that the viability and recovery of engrafting cells is substantially maintained upon freezing and thawing. In particular, if cells are resuspended at a low concentration the number of viable cells recovered after freezing greatly decreases. On the other hand, if the cells are resuspended at a high concentration they may form clumps, thereby limiting the number of recoverable cells. Thus, for purposes of the present invention the engrafting cells should be resuspended to a concentration of about 1×10^6 to 100×10^6 cells/ml, and preferably to a concentration of about 10×10^6 to about 40×10^6 cells/ml.

The resuspended cells are then frozen under a first set of conditions which substantially maintain cell viability. Briefly, if cells are frozen too rapidly, ice nucleation begins the formation of ice crystals which can rupture the cells. Alternatively, if cells are frozen too slowly, dehydration of the cell results. Therefore, in one embodiment the cells are frozen at a controlled rate. This method is particularly preferred for solutions which do not contain HES. Since it is often difficult to maintain a controlled rate of freezing near -4°C (at this temperature cells give off heat termed the "latent heat of fusion"), a controlled rate freezer such as CryoMed's Model 1010 (CryoMed, New Baltimore, Mich.) which provides 'burst cooling' near -4°C may be utilized. As shown in Figure 1, a burst of cooling is provided by the freezer at about -4°C in order to maintain the constant rate of temperature drop in the cell suspension.

Within a particularly preferred embodiment, freezing comprises the steps of (a) cooling the cells down to about 4°C, (b) cooling down the 4°C cells at a rate of about 1.0 degree per minute until the cells reach about -4°C, (c) cooling down the 4°C cells at a rate of about 0.5 degrees per minute until the cells reach about -20°C, (d) cooling down the -20°C cells at a rate of about 1.0 degree per minute until the cells reach about -40°C, and (e) cooling down the -40°C cells at a rate of about 10.0 degrees per minute until the cells reach about -90°C.

It should also be noted, however, that other conditions may be utilized to freeze the engrafting cells. For example, cells which were resuspended in a solution containing about 6% HES may be frozen by placing them directly into an -85°C freezer. Subsequently, if desired, they may be placed into liquid nitrogen.

The resuspended cells may be frozen in any sterile vial suitable for storage in liquid nitrogen. Particularly preferred are Cryotubes (Corning Glass Works, Corning, N.Y.). Alternatively, if desired, the engrafting cells may also be directly frozen in a syringe. Freezing purified engrafting cells in a syringe is particularly advantageous because the same container can be used for both freezing and administration to a patient. This limits possible contamination or loss of cells and increases the speed with which the recently thawed cells can be given to a patient. Alternatively, the cells may also be frozen in a freezing bag, commercially available from Fenwac or Delmed.

As noted above, subsequent to freezing, the cells are thawed under a second set of conditions which substantially maintain cell viability. In a preferred embodiment, the cells are thawed rapidly, preferably in a 37°C water bath.

Once the cells have been thawed, they may be diluted in order to reduce the concentration of a penetrating cryoprotectant or other excipients, and also in order to return the cells to their normal state. For example, cells which were equilibrated with a solution containing 10% DMSO are at an osmolarity of 1800 mosm, while physiological fluids are at an osmolarity of 300 mosm. If the thawed cells are placed immediately into a physiologic solution, the cells would quickly absorb water to equalize the osmotic pressure. This rapid absorption of water results in the lysis of many cells. Therefore, slow dilution of the thawed cells with a physiological buffer, such as PBS, is particularly preferred because it allows time for water and dissolved compounds to equilibrate across the cell membrane, thereby limiting the dangerous swelling of the cells. A particularly

preferred method for reducing the concentration of DMSO is set forth below in Example 6.

Administration of Purified Engrafting Cells

A patient may be immunocompromised for a variety of reasons. For example, a patient may be immunocompromised due to inherent genetic abnormalities, due to disease, or due to the use of toxic chemicals or irradiation in the treatment of cancer. Such a patient may be treated by the administration of a composition comprising a therapeutic dose of engrafting cells, and an aqueous solution containing a total of about 0.002 ml to about 1 ml of DMSO. Within the context of the present invention, a "therapeutic dose" of engrafting cells refers to the number of [CD 34 positive] cells necessary to reconstitute a patient's immune response. The number of cells required ranges from about 0.1×10^6 cells/ml/kilo to about 20×10^6 cells/ml/kilo, although at least 0.75×10^6 cells/ml/kilo (of patient weight) is particularly preferred. Thus, for a patient that weighs 100 kilos, approximately 75×10^6 cells may be administered. The cells should be transferred intravenously from a drip bag, or by direct injection from a syringe. An example of this procedure and the resultant benefits is described in more detail below in Example 11.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1
Preparation Of An Avidinated Biogel

A. Carboxylation of a Polyacrylamide Gel

Seventeen grams of dry Biogel P-60™, (50-100 mesh (wet), coarse beads) (BIORAD, Catalog No. 150, 1630, Richmond, Calif.) are added to 1.5 l of 0.5 M $NaHCO_3$/0.5 M Na_2CO_3. The pH is adjusted to 10.5 with NaOH and carefully stirred with a mixer (RZR1, Carfamo, Wiarton, Ontario, Canada) so as not to damage the beads for approximately 20 to 30 minutes. The mixture is then placed in a 60°C water bath. After the mixture reached a temperature of 60°C, it is incubated for an additional 2 hours (at 60°C) with occasional stirring. The

mixture is then removed from the water bath, and placed in an ice bath to bring the mixture temperature down to room temperature.

The beads are washed several times with distilled or deionized water, followed by several washings with PBS using a coarse glass filter connected to a vacuum. The carboxylated gel may be stored in PBS at 4°C, and is stable for up to one year if sterilized or stored with a preservative.

B. Avidin Conjugation of Carboxylated Biogel

PBS is first removed from a measured amount of carboxylated Biogel by filtering with a coarse glass filter connected to a vacuum. The gel is then equilibrated in distilled or deionized water for 15 to 30 minutes. Equilibration in water causes an expansion of the gel to a volume of about 4 times its previously measured amount. The gel is resuspended in 10 ml of distilled or deionized water for each ml of gel (as originally measured in PBS).

Thirty mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCl) (Sigma Chemical Co., Catalog No. E7750, St. Louis, Mo.) is added for each ml of gel as originally measured. The pH is rapidly adjusted to 5.5 by dropwise addition of HCl. Care is taken to maintain the pH at 5.5; pHs of less than 5.0 or greater than 6.0 result in significantly less activation of the Biogel. The mixture is stirred for five minutes.

Avidin (International Enzymes, Inc., Fallbrook, Calif.) is dissolved at a concentration of between 10 and 100 mg/ml in deionized water. Next, 1 mg of avidin is rapidly added for each ml of gel (as originally measured in PBS). The mixture is stirred for 1.5 hours. Next, 2 M glycine is added to give a final concentration of 0.2 M glycine in the mixture, and stirred for an additional 1 hour.

The gel is washed with several volumes of PBS using a coarse glass filter and vacuum, and stored in PBS at 4°C. The gel is stable for approximately one year.

EXAMPLE 2

Isolation Of Engrafting Cells

A. Preparing the Buffy Coat Cells

A sample of bone marrow is centrifuged at 240 x g for 15 minutes. The plasma is removed (and is retained for later use), and the remaining buffy coat cells are centrifuged once more at 240 x g for 15 minutes in order to remove red blood cells. The buffy coat cells are washed twice with RPMI by

centrifugation at 180 x g for 10 minutes. The cells are then resuspended to a final concentration of 1×10^8 white cells/ml in RPMI plus 1% BSA.

B. Incubation of Buffy Coat Cells With Antibody

The suspension of buffy coat cells is incubated with a final concentration OF 20 µg/ml biotinylated anti-CD 34 antibody (CellPro®, Bothell, Wash.) at room temperature for 25 minutes. The antibody-cell mixture is then washed twice with PBS by centrifugation at 180 x g for 10 minutes. The cells are then resuspended at a concentration of 1×10^8 white cells/ml in PBS.

C. Column Operation and Results

A CEPRATE LC™ (CellPro®, Bothell, Wash.) separating system was utilized essentially according to the manufacturer's instructions. Briefly, the instrument was set up, the tubing connected, reagent's were loaded, and the process run was begun with the antibody treated cells. The cells were pumped through the column, the column was washed with PBS, then the adsorbed cells were released via the magnetically driven impeller. The adsorbed cells were accumulated in a collection bag.

D. Results

Ten billion bone marrow cells were passed through the column; 200 million of the cells were bound to the column and were recovered in the collection bag. Viability of the collected cells was 91% as measured by trypan blue exclusion. The collected cells were 75% CD 34^+ as measured by FACS analysis.

EXAMPLE 3

Concentration of Engrafting Cells

The collection bag containing purified engrafting cells is gently inverted in order to mix the cells. The cells are then transferred into two sterile 50 ml centrifuge tubes which have been coated with autologous plasma. Thirty milliliters of TC 199 is used to rinse out the cell collection bag. The rinse volume is placed into a third 50 ml tube. The bag is then rinsed a second time with 20 ml of TC 199, which is also added to the third 50 ml tube. The three tubes are centrifuged at 150 x g for 10 minutes.

EXAMPLE 4

Resuspension of Engrafting Cells

Supernatant is removed from the concentrated engrafting cells. One milliliter of engrafting cell media (TC 199, 40% autologous plasma and 10 U/ml heparin) is added to each of the two tubes with large pellets, and combined into the small pellet rinse tube. This yields approximately 2.25 mls of cell suspension which has a concentration of 50×10^6 to 100×10^6 cells/ml.

TC 199 containing 10 u/ml of heparin is added to the cell suspension to bring the final volume to 4.5 mls. Then, 0.9 ml of autologous plasma and 0.34 ml of DMSO is added.

An equal volume of cryoprotective media (TC 199 with 15% DMSO) is added gradually to the cell suspension. Four and a half ml is aliquoted into each Cryotube and placed in the "precooled chamber" of a CryoMed controlled rate freezer. A "dummy" tube with an identical volume of cryoprotective media is prepared and placed in the freezer. A thermocouple is placed in the tube in order to record the freezing rate in the tube.

EXAMPLE 5

Freezing Engrafting Cells

As shown in Figure 1, the engrafting cells are frozen at a controlled rate from room temperature to -90°C. Briefly, this method is comprised of the following steps: (a) cooling the cells down to about 4°C, (b) cooling down the 4°C cells at a rate of about 1.0 degree per minute until the cells reach about -4°C, (c) cooling down the -4°C cells at a rate of about 0.5 degrees per minute until the cells reach about -20°C, (d) cooling down the -20°C cells at a rate of about 1.0 degree per minute until the cells reach about -40°C, and (e) cooling down the -40°C cells at a rate of about 10.0 degrees per minute until the cells reach about -90°C. As shown in Figure 1, this protocol results in a controlled rate of freezing.

EXAMPLE 6
Thawing Engrafting Cells

The cryotube containing purified engrafting cells is removed from liquid nitrogen, and placed in a sterile ziplock bag. The bag containing the tube is placed in a 37°C water bath and agitated. As the last ice crystal dissolves the contents are transferred to a 50 ml centrifuge tube.

Four and one-half milliliters of warmed engrafting Cell Dilution Media (TC 199 and 10 u/ml heparin) is added slowly (one drop at a time initially) into the 50 ml tube. Addition of this media should take approximately 2 minutes. Following the first dilution subsequent volumes of 0.5 mls of engrafting Cell Dilution Media may be added followed by gentle mixing of the cells, until a final volume of 30 ml has been attained.

EXAMPLE 7
Comparison of Cryopreserved Engrafting Cells and Cryopreserved Bone Marrow

Engrafting cells were purified and cryopreserved as described above in Examples 2 through 6. Buffy coats were also prepared from bone marrow and treated similar to the engrafting cells, except that they were not purified. As shown in Figure 2, purified engrafting cells were significantly more viable, and significantly more total cells (and CFCs) were recovered than for whole marrow.

EXAMPLE 8
Determination of CFC Viability and Recovery

One ml per 35 mm plate of Iscove's Methylcellulose (Terry Fox Laboratories, Vancouver, British Columbia, Canada) supplemented with 2 mM L-glutamine and 50 mg/ml gentamicin was warmed to 37°C. Cells were plated in triplicate at 3-fold dilutions to improve the accuracy of the assay. The highest number of cells plated was 10^5/plate except for purified cells which were plated at 3×10^3 and less. The cells were spread evenly over the surface of each plate and then incubated in a humidified incubator at 37°C with 5% CO_2 in air for 10 to 14 days. Colonies were counted if they contained more than 50 cells and scored as CFU-GM, BFU-E, or other (*e.g.*, CFU-GEMM). The number of various types of colonies were summed to give the total number of colony-forming cells (CFC).

EXAMPLE 9

Effect of Cell Concentration on Cryopreservation

Engrafting cells were purified and cryopreserved as described above in Examples 2 through 6, except that the cells were frozen at different concentrations including: 2.5, 5, 10, 25, 40, and 50 million cells/ml. As shown in Figure 3, CFC recovery was the greatest when cells were frozen at a concentration ranging from 10×10^6 to 40×10^6 cells/ml.

EXAMPLE 10

Effect of DMSO on Cryopreservation

Engrafting cells were purified and cryopreserved as described above in Examples 2 through 6, except that the cells were frozen at a final concentration of 7.5% and 10% DMSO. As shown in Figure 4, cell viability, recovery, and CFC recovery were significantly better when only 7.5% DMSO was utilized.

EXAMPLE 11

Administration of Engrafting Cells

Four patients with breast cancer had bone marrow removed by iliac crest aspirations. They were subsequently treated by chemotherapy in order to kill the tumor.

Engrafting cells were purified essentially as described above from the patent's marrow, and administered to the patients. The results of this therapy is set forth below in Table I.

TABLE I

Patient Wt. (kg)	#CD 34+ Infused (Million/kg)	Days to Granulocyte Recovery	Days to Platelet Recovery	# Units Transfused Platelets
59.8	.98	25	24	13
84.6	1.64	17	36	9
51	4.32	13	16	3
57	1.05	32	30	22

Typically, engraftment (the number of days it takes a patient's granulocyte counts to rise above 500 cells/μl) takes approximately 20 to 35 days. The above patients which were treated with purified engrafting cells demonstrated engraftment after only 13 to 32 days. In addition, as noted above, the total volume of DMSO which was infused into the patients differs markedly from conventional procedures. In particular, only about 0.002 ml to about 1 ml of total DMSO (0.675 ml) was infused into each patient. Cell aggregates and hemoglobin were not detected in the cells to be reinfused. Thus, these patients demonstrated none of the toxicities which are typically associated with marrow reinfusion, such as nausea, headache, chills, dizziness, vomiting, heart arrhythmia, hypertension, pulmonary distress, or renal failure.

Moreover, patients which receive autologous bone marrow transplants often experience delayed platelet recovery and require a substantial number of platelet transfusions (on average about 30). The patients who received the above-described therapy, however, required only 12 units of platelets each (on average). This reduction in platelet usage decreases the potential of blood-borne disease transmission, the risk of alloimmunization, and the cost (each unit of platelets costs the patient approximately $300). Thus, it can be seen that the present invention provides substantial advantages over previously available cryopreservation techniques.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A method for preparing engrafting cells for future use comprising:

(a) purifying engrafting cells from a suitable blood product;

(b) concentrating the purified engrafting cells;

(c) resuspending the concentrated cells to a concentration of about 10×10^6 to 40×10^6 cells/ml in a solution that substantially maintains cell viability during freezing and thawing; and

(d) freezing the resuspended cells under a set of conditions which substantially maintains cell viability.

2. A method for preparing engrafting cells for future use comprising:

(a) purifying engrafting cells from a suitable blood product;

(b) concentrating the purified engrafting cells;

(c) resuspending the concentrated cells in a solution that substantially maintains cell viability during freezing and thawing, said solution containing a total of 0.002 ml to about 1 ml of DMSO; and

(d) freezing the resuspended cells under a set of conditions which substantially maintains cell viability.

3. The method of claims 1 or 2, further comprising, subsequent to the step of freezing, thawing the frozen cells under a set of conditions which substantially maintains cell viability.

4. The method of claim 3 wherein said cells are thawed in a 37°C water bath.

5. The method of claim 3 further comprising, subsequent to the step of thawing, diluting the thawed cells slowly with a physiological buffer.

6. The method of claim 1 wherein the step of purifying comprises passing the blood product over an immunoaffinity column which purifies the engrafting cells.

7. The method of claim 1 wherein the step of concentrating comprises centrifuging the engrafting cells.

8. The method of claim 2 wherein the concentrated engrafting cells are resuspended to a concentration of about 10×10^6 to about 40×10^6 cells per ml.

9. The method of claims 1 or 2 wherein said solution contains about 6% HES.

10. The method of claim 1 wherein said solution contains a total of about 0.002 ml to about 1 ml of DMSO.

11. The method of claims 1 or 2 wherein said solution comprises media, protein, and a penetrating cryoprotectant.

12. The method of claim 11 wherein the media is selected from the group consisting of RPMI 1640, TC 199, and Iscoves DMEM.

13. The method of claims 1 or 2 wherein the step of freezing comprises freezing the cells at a controlled rate.

14. The method of claim 13 wherein the step of freezing at a controlled rate comprises:

(a) cooling the cells down to about 4° C;

(b) cooling down the 4°C cells at a rate of about 1.0 degree per minute until the cells reach about -4°C;

(c) cooling down the -4°C cells at a rate of about 0.5 degrees per minute until the cells reach about -20°C;

(d) cooling down the -20°C cells at a rate of about 1.0 degree per minute until the cells reach about -40°C; and

(e) cooling down the -40°C cells at a rate of about 10.0 degrees per minute until the cells reach about -90°C.

15. A composition comprising:

(a) a therapeutic dose of engrafting cells; and

(b) an aqueous solution containing a total of about 0.002 ml to about 1 ml of DMSO.

16. A method for treating immunocompromised patients comprising administering to said patient the composition of claim 15.

17. A syringe containing the composition of claim 15.

18. A drip bag containing the composition of claim 15.

FIGURE 1

FREEZING CURVE

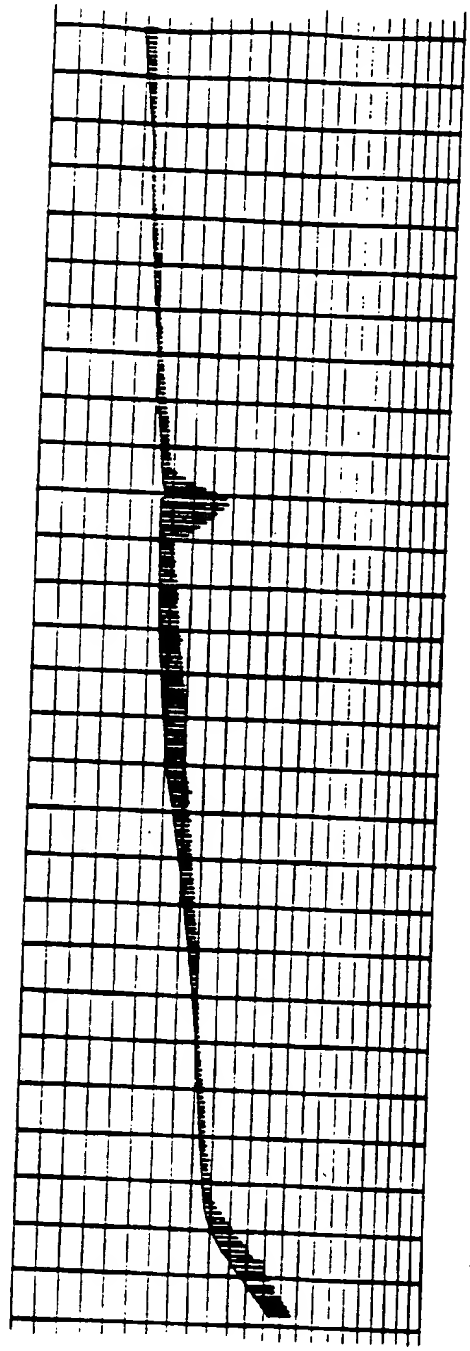


FIGURE 2

COMPARISON OF CRYOPRESERVED ENGRAFTIN CELLS AND CRYOPRESERVED WHOLE MARROW

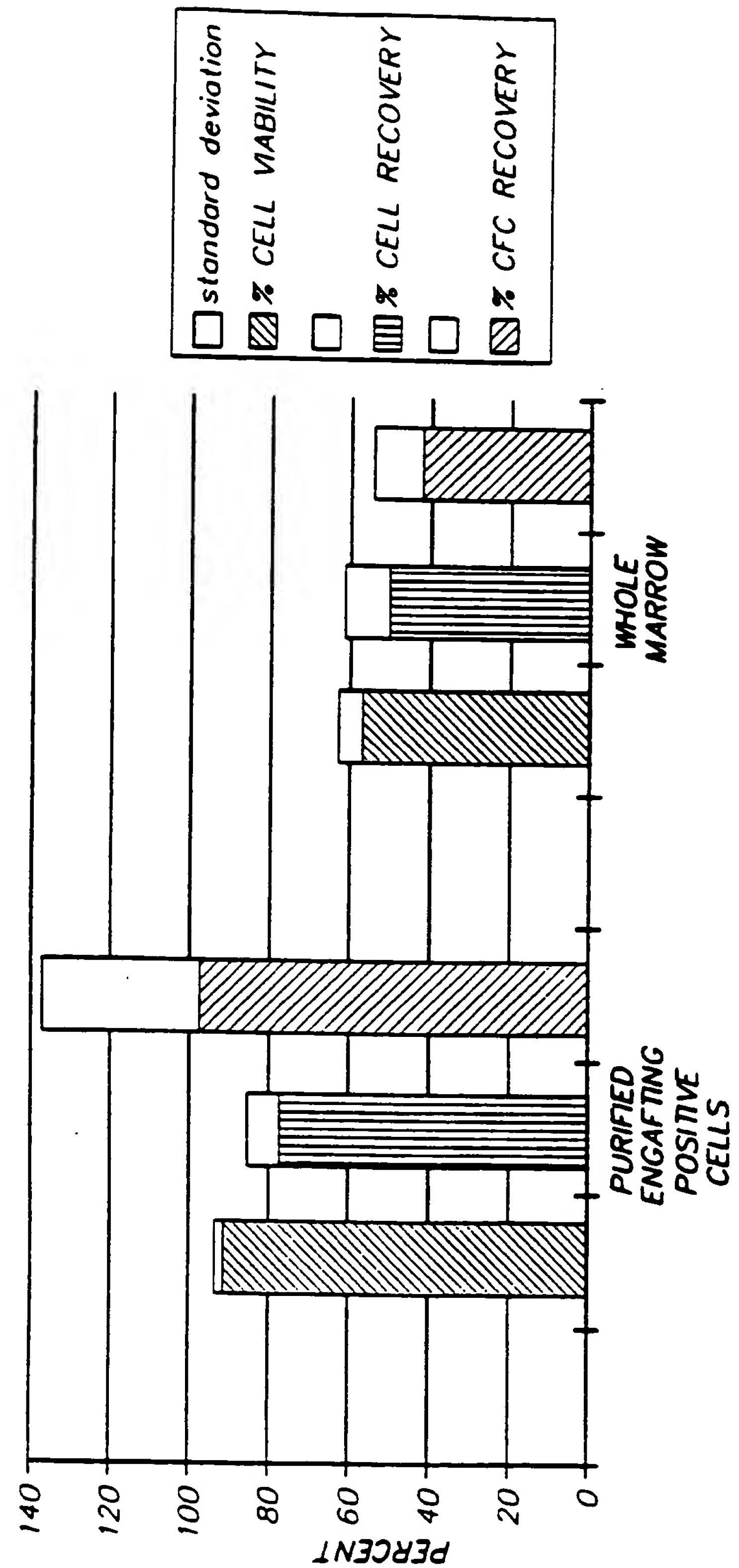
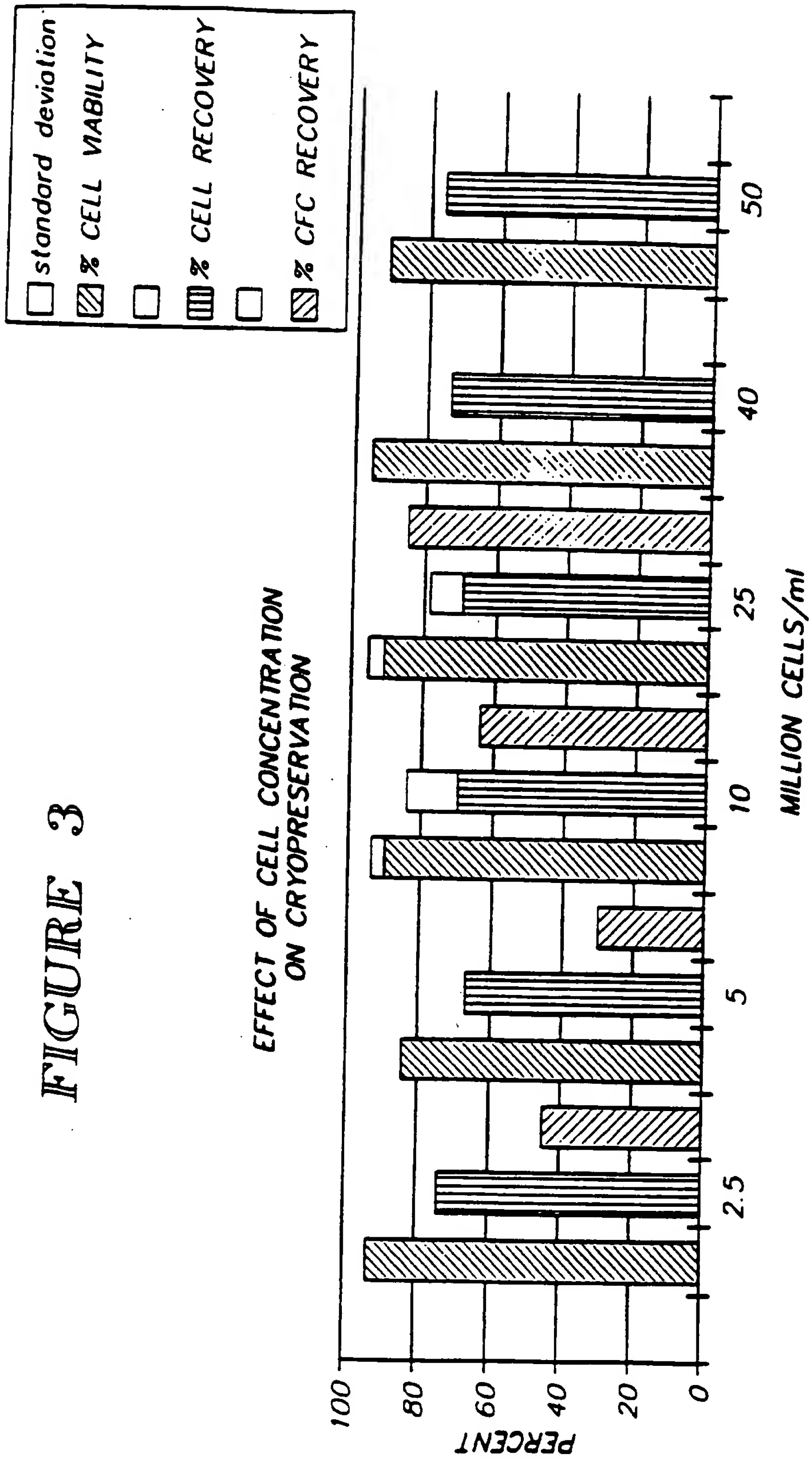
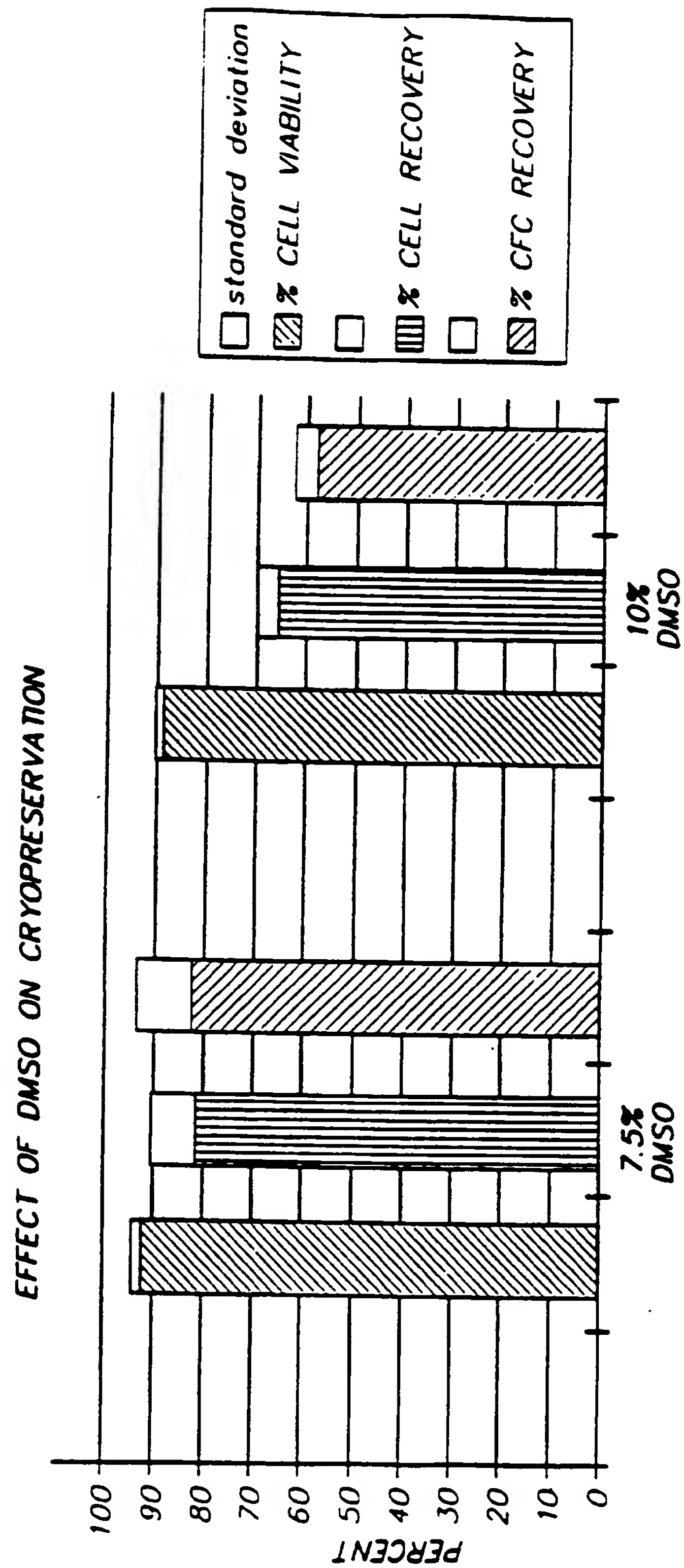


FIGURE 3



EFFECT OF CELL CONCENTRATION ON CRYOPRESERVATION

FIGURE 4



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09023

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A01N1/02; A61K35/28; A61K35/14

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C12N ; A01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,8 904 168 (BIOCYTE CORPORATION) 18 May 1989 see page 37, line 1 - page 47, line 17; claims 1-9,14-26; examples 6.4-6.6,6.9	1-18
X	EP,A,0 451 611 (SYSTEMIX, INC.) 16 October 1991 see page 3, line 7 - line 15; claims 1-3,6-8 see page 5, line 50 - page 6, line 52	1-18

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 JANUARY 1993	5. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.O.

Form PCT/ISA/210 (second sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/09023

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 16 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9209023 SA 66078

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/01/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8904168	18-05-89	US-A-	5004681	02-04-91
		AU-A-	2610288	01-06-89
		EP-A-	0343217	29-11-89
		JP-T-	3501207	22-03-91
EP-A-0451611	16-10-91	US-A-	5061620	29-10-91
		AU-A-	7398691	03-10-91

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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